OnLine Journal of Biological Sciences

Original Research Paper

Analysis of Nucleoli in Intact Lymphocytes in Different Mammalian Species and Hybrids

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Article history
Received: 12-09-2021
Revised: 23-11-2021
Accepted: 22-12-2021

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Abstract: The purpose of this study was to develop methods for quantitative and qualitative assessment of the activity of the nucleolus using the color intensity of argyrophilic regions and to study the effect of the species feature on the quantitative and qualitative indicators of the nucleoli and nucleolus organizer regions. The experiment involved *Ovis aries* (n = 40), hybrids of domestic sheep and argali (n = 10), Capra hircus (n = 32), *Myocastor coypus* (n = 25) and the American mink neogale vison (n = 20). To characterize the activity of the nuclear apparatus of interphase cells, the number of argyrophilic zones in the AgNOR cell was determined; the total area of the nucleoli ΣS_{NOR} expressed in logical units, and the color intensity of the nucleus and its various regions in logical units. Microsoft excel-2010 software was used to process the obtained primary digital materials and the SPSS v.23.0 software package was used for statistical processing. The number of nucleolus organizer regions, depending on the species and individual characteristics, in representatives of the genus Ovis varied from 0 to 9 and averaged 3.07±0.19 NOR per cell, in the genus Capra 2.74±0.12. The maximum number of NOR in the interphase cells of lymphocytes of the studied population of a domestic goat was 8, the minimum number was the absence of visualized NOR. The coefficient of variation of the number of NOR in this population was 52%. In the interphase lymphocytes of the American mink, the frequency of occurrence of NOR averaged 0.84±0.01, the maximum number was 4, and the minimum number was the absence of visible crossed NOR, in nutria, it was 0.63±0.01 and 2.0, respectively. The minimum value of the indicators characterizing the parameters of the nucleolar area and the color density of argyrophilic zones (D_{NOR}) was established by *Myocastor coypus*. The results of the study show that one of the main factors affecting the parameters of the nucleolus is the specific feature of the organism.

Keywords: Nucleolus Organizer Regions (NOR), Ribosomal RNA Genes (rRNA Genes), Computer Analysis, Nucleolus, Lymphocytes, Nucleolar Organizer (NO), Hybrids

Introduction

The nucleolus is the largest sub-compartment of the nucleus of higher eukaryotes. It is a membraneless structural unit whose function is the synthesis of ribosomal RNA (rRNA) and ribosome biogenesis (Bersaglieri and Santoro, 2019; Bilban and Vaupoti 2001; Boisvert *et al*., 2007). In the genomes of eukaryotes, copies of rRNA genes are abundant. Arrays of tandem repeats of the copies of rRNA genes are located in different chromosomes of the so-called Nucleolus Organizer Regions (NORs). Transcription of the rRNA gene generates 45S/47S pre-rRNA, which is then modified to form 28S, 18, and 5.8S rRNAs (Boulon *et al*., 2010).

The amount of RNA in the nucleolus is one of the most important factors influencing the formation of the nuclear structure. The nuclear scaffold protein is formed from the

nucleolus RNA (Chen *et al*., 1998). The nucleolus is a dynamic structure; its activity depends on biotic and abiotic factors (Chen and Pikaard, 1997; Correll *et al*., 2019; Derenzini *et al*., 2009). The nucleolus plays an important role in the regulation of the genome. Inhibition of rRNA transcription leads to a decrease in its level in the nucleolus as a result of changes in the structure and composition of the nucleolus. In the process of inhibition of rRNA transcription, there is a protein shift; one group of proteins is shifted to the periphery and the other to the nucleoplasm. A group of peripheral proteins from the nucleolar cap. The protein shift leads to a change in the composition of the nucleolus and its architecture (Derenzini *et al*., 1985; 1998). The nucleolus is one of the main subunits of the cell that regulate its life cycle (Donizy *et al*., 2017; Donjerkovic, 2000; Earley *et al*., 2006). It plays an important role in ribonucleoprotein biogenesis, regulation, and telomerase activity (Fox, 2010; Grob *et al*., 2009). The size of the nucleolus depends on the species and can be less than 1 µm and more than 10 µm, for example, the size of the nucleolus in yeast is less than 1 µm, while in peas it is more than 10 µm. The mechanism for controlling individual sizes of cell subunits is not fully understood and is of great fundamental importance (Hiscox, 2007).

One of the factors affecting the size of the nucleolus is the intensity of rRNA synthesis, there is a positive correlation between these parameters (Howell and Black, 1980; Howell *et al*., 1975). The number of rRNA increases in cells in the cell-division cycle and the nucleolus increases accordingly in such cells (Iarovaia *et al*., 2019). The number of NOR in interphase cells correlates with the proliferative activity of cells. Studies show that during the ontogenetic development of the organism, the NOR index in the interphase nuclei of lymphocytes decreases as a result of a decrease in proliferative and metabolic processes in cells.

The state of the nucleolar apparatus is one of the criteria for assessing the activity of the cell in various physiological and pathological processes. Increased regulation occurs in response to external mitogenic signals that promote cell growth and proliferation, while reduced regulation occurs in conditions that disrupt cellular metabolisms, such as nutrient depletion, genotoxicity, and oxidative stress (Lawrence and Pikaard, 2004; Lindström *et al.*, 2018; Ma *et al*., 2016). Ribosomal genes play an important role in the process of adaptation of the organism to unfavorable environmental conditions (Mangan *et al*., 2017; Matera *et al*., 2007; McStay, 2016).

Ribosomal genes prevent the accumulation of active oxygen, providing antioxidant protection and preventing DNA damage. Clusters of ribosomal genes are localized in the short arms of acrocentric chromosomes (Miller *et al*., 1976). The number of Nucleolus Organizer Regions (NORs) and active clusters of ribosomal genes have a large variability depending on intercellular and individual characteristics. The nucleolus contains acidic non-histone proteins (C23, B23, UBF, and RNA polymerase), which are characterized by specific staining with silver nitrate (Miroslav *et al*., 2017; Pelletier *et al*., 2018; Pontvianne *et al*., 2012). These proteins are responsible for the activation and control of the transcription of ribosomal genes localized in the NOR. The association of these proteins with the quantitative parameters of argyrophilic structures can be used to assess the activity of ribosomal genes (Preuss *et al*., 2008; Schöfer *et al*., 2018). The intensity of Ag-NOR staining used to detect acidic non-histone proteins of NOR can be used to assess their activity. The activity of the nucleolus makes it possible to assess the proportion of AcRG on metaphase chromosomes and serves as a fairly stable trait (Sirri *et al*., 2000). It has been shown that the introduction of double-strand breaks in rDNA (using genome editing technologies or laser micro-radiation) can lead to a radical restructuring of the nucleus structure, ATM-dependent transcription suppression, and activation of various repair mechanisms, which indicates the important role of rDNA in maintaining the structural integrity of the genome.

The purpose of the study is to develop methods for quantitative and qualitative assessment of the activity of the nucleolus using the color intensity of argyrophilic regions and to study the effect of the species feature on the quantitative and qualitative indicators of the nucleoli and nucleolus organizer regions.

Materials and Methods

Legal Requirements

The protocols of experiments were approved by the Committee for Animal Care and Use of the L.K. Ernst Federal Research Centre for Animal Production per Decision No. 80 of the Council of the Eurasian Economic Commission of 10 November 2017 "On Approval of the Rules for Organization of Laboratory Testing during Veterinary Control (Supervision)".

Animals

The experiment involved domestic sheep (*Ovis aries*) (n = 40), hybrids of domestic sheep and argali (n = 10), domestic goat (Capra hircus) (n = 32), nutria (*Myocastor coypus*) (n = 25) and American mink (*neogale vison*) (n = 20). The groups included animals of different ages and physiological conditions. All the animals were examined by the veterinary service.

Collecting Blood and Preparing Slides

Blood from animals was taken from a vein. Lymphocytes were isolated from the blood using the sedimentation method in the gradient of Ficoll-Urographin density. 1 mL of diluted blood was layered into a centrifuge tube for 1 mL of Ficoll solution (ρ = 1.077 g/mL) and centrifuged for 45 min at 3000 rpm. The lymphocyte layer was carefully collected over the entire cross-sectional area of the tube, transferred to a clean, dry centrifuge tube, and diluted with PBS in a ratio of 1:1. The contents of the tube were centrifuged for 5 min at 3000 rpm. Then the supernatant was removed and the resulting precipitate was resuspended in PBS. A smear was prepared and dried at room temperature. The preparations were fixed with methyl alcohol. Nucleolar Organizers (NOs) were detected by staining cells with silver nitrate, carried out according to the method of Howell and Black (1980). At least 50 colored cells from each animal were examined.

Microscopy the resulting slides were examined using a Nikon Eclipse Ni microscope equipped with a DS-Qi2 digital video camera. Measurements and processing of the resulting images were carried out using the NIS-Elements BR4.30 and Image Scope 1.0 software.

Measurement of the Parameters of the Nucleoli

The average color intensity of the nucleoli and their total area were determined.

To characterize the nucleolar apparatus, the following parameters were used, determined in each cell:

- Ag_{NOR}-the number of argyrophilic zones in the cell
- ΣS_{NOR} is the total area of the nucleoli expressed in logical units
- The intensity of the color of the nucleolus and its various regions in logical units

The intensity (density) of the color was determined by the formula:

D = 254-F, where
D = Color density
254 = The number of logical units corresponding to the white color
F = The average value of the object's brightness in logical units

The average color density of argyrophilic zones (D_{NOR}), as well as the average color density of the nucleolus (D_N) and its areas free of NORs (D_F) were determined. To characterize the argyrophilic zones of cells, the extinction value was also determined by the formula:

$$EXT_N = D_N - D_F, \text{ where}$$

EXT_N - Extinction of argyrophilic zones
D_N - Average color density of the nucleolus
D_F - Average color density of the areas free from NOR

As an integral estimate, the optical equivalent of the NOR was calculated:

$$OE_{NOR} = D_{NOR} x \Sigma S_{NOR}, \text{ where}$$

OE_{NOR} - Optical equivalent of NOR
D_{NOR} - Color density of argyrophilic zones
ΣS_{NOR} - Total area of nucleoli

To determine the value of D_{NOR}, the formula was used:

$$D_{NOR} = \left(S_N \times D_N - S_F \times D_F\right) / \Sigma S_{NOR}, \text{ where}$$

S_N - Nucleolus area
D_N - Average nucleolus density
S_F - The area of the nucleolus-free core zone
D_F - Average density of the nucleolus-free zone

This approach makes it possible to speed up the analysis of argyrophilic structures, eliminating the need to measure the parameters of each nucleolus, while simultaneously reducing the measurement error for ΣS_{NOR} and D_{NOR}.

The index of nucleolar activity was determined, which is expressed by the ratio of cells with nucleoli to the total number of counted cells.

Statistics

Microsoft Excel-2010 software was used to process the obtained primary digital materials and the SPSS v.23.0 software package was used for statistical processing.

Novelty

To characterize the activity of the nuclear apparatus of interphase cells, the number of argyrophilic zones in the AgNOR cell was determined; the total area of the nucleoli ΣS_{NOR} expressed in logical units, and the color intensity of the nucleus and its various regions in logical units.

Results

The results of the analysis of colored slides show that the number and localization of Nucleolar Organizers (NO) has a specific feature. In *Ovis aries*, they are localized on the short arms of 1 pair of chromosomes, the long arms of 2 and 3 pairs, and in the telomeric regions of the long arms of the acrocentric chromosomes of 4 and 25

pairs. In the diploid set of chromosomes of the genus *Capra*, a set of 18/28S rRNA genes are localized in the NOR, just like in sheep in 10 clusters. They are located in the telomeric regions of 2, 3, 4, 5, and 28 pairs. The nucleolar organizers of *neogale vison* are located on 2 and 8 pairs of chromosomes. On one or two chromosomes of the 2nd pair, gene clusters are in an active state. The nucleolus organizer regions in *Myocastor coypus* are localized in secondary constrictions on 19 pairs of chromosomes.

The number of nucleolus organizer regions, depending on the species and individual characteristics, in representatives of the genus *Ovis* varied from 0 to 9 and averaged 3.07±0.19 NOR per cell, the mode and median in representatives of *Ovis* were 3, in the genus *Capra* 2.74±0.12 (Table 1). The maximum number of NOR in the interphase cells of lymphocytes of the studied population of a domestic goat was 8, the minimum number was the absence of visualized NOR. The coefficient of variation of the number of NOR in this population was 52%. The median and mode for this indicator were 2. The most common cells were those with 2 NOR. In the interphase lymphocytes of the American mink, the frequency of occurrence of NOR averaged 0.84±0.01, the maximum number was 4, and the minimum number was the absence of visible crossed NOR, in nutria, it was 0.63±0.01 and 2.0, respectively (Fig. 1).

The results of the study show that in the studied population, the representatives of the genus Ovis and Capra have an advantage over the representative of *neogale vison* and *Mycastor coypus* in terms of the number of argyrophilic zones, the difference between the groups is statistically significant ($p<0.001$).

The maximum index of nucleolar organizers was found in hybrids of domestic argali sheep and amounted to 2.85, the minimum value is typical for nutria and amounted to 0.65 (Fig. 2). The index value depends on the number of NORs. The difference between units is significantly different and reliable ($p<0.001$).

The area of argyrophilic zones is highly variable. The average total area of nucleoli in *Ovis aries* averaged 263.42±17.28 pix, while in their hybrids with *O. ammon* it averaged 285,32±14,26 pix and 216,18±12,30 in *Capra* goats. The difference between species and hybrids belonging to the subfamily *Caprinae* in terms of area and color intensity is not statistically significant (Table 2).

The minimum value of the indicators characterizing the parameters of the nucleolar area and the color density of argyrophilic zones (D_{NOR}) was established by *Myocastor coypus*. The difference between the species belonging to different families is statistically significant ($p<0.001$).

Table1: NOR content in interphase cells of different species and hybrids

	Species				
Parameters	Ovis aries	*O. aries* x *O. ammon*	Capra hircus	Neogale vison	*Myocastor coypus*
Average	3.07[ab]	3.30[ab]	2.74[ab]	0.84	0.63
Standard error	0.190	0.230	0.120	0.01	0.03
Median	3.000	3.000	2.000	1.00	1.00
Mode	3.000	3.000	2.000	1.00	1.00
Standard deviation	1.590	1.830	1.430	0.16	0.18
Sample variance	2.530	2.850	2.050	0.68	0.47
Excess	2.320	2.670	1.260	0.85	0.72
Asymmetry	1.360	1.610	1.060	0.75	0.65
Interval	9.000	10.000	8.000	4.00	2.00
Minimum	0.000	0.000	0.000	0.00	0.00
Maximum	9.000	10.000	8.000	4.00	2.00

[a]the mean is compared with neogale vison, [b]the mean is compared with *myocastor coypus*. Diacritical marks shows the differences between species. Significance level of difference between species ($p<0.001$).

Table 2: Parameters of the nucleolus of interphase cells in different species

Species	Nucleolus area, pix	Total area of nucleoli, pix	The proportion of the area of the nucleoli in the nucleus, %	OE_{NOR}	D_{NOR}
Ovis aries	4318.3±93.5[ab]	263.4±17.28[ab]	6.10±0.4[ab]	41025±1012	194.83±9.3[ab]
O. ariesx O. Ammon	4837.7±76.2[ab]	297.5±19.35[ab]	6.15±0.8[ab]	75619±2025	210.05±8.6[ab]
Capra	4293.4±83.4[ab]	252,2±17.4[ab]	5.87±0.5[ab]	40895±932	191.27±9.5[ab]
neogale vison	3479.5±120.7[c]	195.6±12.3[c]	5.62±0.5[c]	36782±856	134.56±7.3
Myocastor coypus	2835.7±130.5	132.3±14.5	4.66±0.3	31653±563	110.26±9.5

[a]the mean is compared with *neogale vison*, [and b]the mean is compared with *Myocastor coypus*

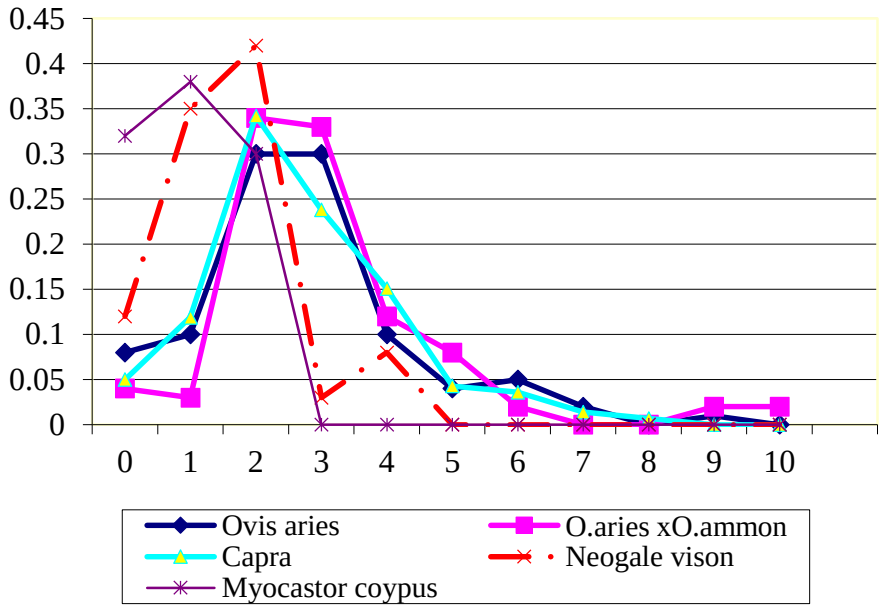

Fig. 1: Frequency of NOR in interphase lymphocytes of different species

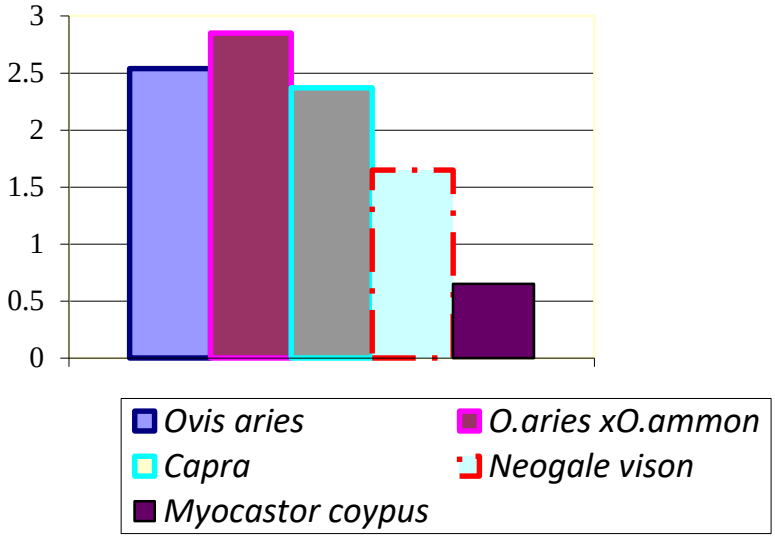

Fig. 2: Indicators of the index of nucleolar organizers in different species and hybrids

Discussion

One of the main criteria for assessing the state of the organism at the cellular level is the parameters of the nucleolar apparatus. Cell activity depends on the physiological state of the organism (Wang and Lemos, 2017). External mitogenic factors lead to cell proliferation, and oxidative stress; genotoxicity leads to inhibition of cell reproduction (Xu *et al*., 2017; Correll *et al*., 2019). The main method for evaluating the parameters of cell activity is the number of active Nucleolar Organizers (NO) in the cell, visualized using silver nitric acid staining (Howell and Black, 1980). Currently, the main parameter for evaluating cell activity is the amount of NOR. In recent years, the parameters of the nucleolar apparatus have been widely used for prognostic purposes, especially for cancer diseases (Derenzini *et al*., 2009; Donizy *et al*., 2017; Donjerkovic and Scott, 2000). The results of our research show that qualitative and quantitative indicators differ significantly depending on biotic factors. In this

project, the influence of the species factor on the parameters of the nucleolar apparatus of interphase cells was studied. To evaluate the parameters of nucleoli, various protocols and software are being developed that allow determining the index of the destruction of the nucleolus (Stamatopoulou *et al*., 2018). Our results show that there is a difference between the hybrids of *O. aries*and *O. ammon*compared to the original parent form of *O. aries.* In recent years, studies have found the phenomenon of nucleolar dominance in many interspecific hybrids, including hybrid plants (Chen *et al*., 1998). There is a hypothesis that nucleolar dominance is caused by the suppression of the rRNA gene of one species by another species. The dominance of nucleoli is epigenetic and in hybrids, the expression of 45S rRNA genes occurs only in one of the parents (Tucker *et al*., 2010). The suppression of ribosomal RNA genes leads to a change in DNA methylation and histone modification (Chen and Pikaard, 1997; Lawrence and Pikaard, 2004; Earley *et al*., 2006; Preuss *et al*., 2008; Pontvianne *et al*., 2012).

Conclusion

The results of the study show that the number of nucleoli in the cell nucleus is not enough to assess the activity of nucleoli in interphase cells. For a reliable and comprehensive assessment, it is necessary to take into account qualitative indicators such as the area of NOR and the intensity of their color. Studies show that one of the main factors affecting the parameters of the nucleolus is the specific feature of the organism.

Funding

The survey is conducted under support grant №20-016-00116A, provided by the RFB.

Author Contributions

Baylar Iolchiev: Participated in all experiments, coordinated the data analysis, and contributed to the writing of the manuscript.

Pavel Klenovitskiy: Participated in all experiments, coordinated the data analysis, and contributed to the writing of the manuscript.

Vugar Bagirov: Designed the research plan and organized the study.

Inna Novgorodova: Participated in all experiments, coordinated the data analysis, and contributed to the writing of the manuscript.

Natalya Volkova: Designed the research plan and organized the study.

Neilia Khusnetdinova: Participated in all experiments, coordinated the data analysis, and contributed to the writing of the manuscript.

Prytkov Yuri: Designed the research plan and organized the study.

Anastasia Silanteva: Coordinated the animal work.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and that no ethical issues are involved.

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